Carotenoids Accumulation in the Green Alga *Scenedesmus* sp. Incubated with Industrial Citrate Waste and Different Induction Stresses

El-Sayed, A.B

Fertilization Technology Department, National Research Centre, Dokki, Cairo, Egypt
bokhair@msn.com

Abstract: A laboratory experiment was conducted in the algae-station of the Fertilization Technology Department, National Research Centre, Dokki, Cairo, Egypt with the green alga *Scenedesmus* sp. to study the effect of citrate waste in the nutrient solution on vegetative growth and pigments accumulation. The alga was incubated in 2000 ml culture with 10 ml citric acid waste characterized by high CNPK contents. After maximum growth rate was achieved induction was performed (by the day ten) and separate addition of NaCl, $FeSO_4$ and chelated $FeSO_4$ was performed and citrate volume was raised to be 50ml.l^{-1}. Growth measurements were dry weight, total chlorophyll and carotenoids. Results showed that maximum vegetative growth was reached after approximately 10 days of incubation. The maximum growth rate during this period was 0.29 and 0.26 d^{-1}, while average growth rate was 0.14 and 0.16 $d^{-1}$for dry weight and total chlorophyll, respectively. Except of cultures received chelated $FeSO_4$, all other treated cultures (control, metal $FeSO_4$ and NaCl) resulted in complete degradation of chlorophyll and massive accumulation of carotenoids. The cultures were completely greenish yellow and yellow colored. Maximum chlorophyll ratio was found in control cultures which received citrate only (50ml.l^{-1}), however maximum carotenoids ratio was detected in the algae supplied with metal $FeSO_4$, followed by 2.0% NaCl. [Nature and Science 2010;8(10):34-40]. (ISSN: 1545-0740).

Key words: Citrate wastes; Green algae; Vegetative growth; Carotenoids

1. Introduction

Recycling of particulate organic wastes into marketable production matrix is highly recommended to meet the safe re-use and utilization of wastes which daily increased due to the high industrial development (Stirling and Okumug, 1995). A promising technique in this field is the leaching with microorganisms. With this technique both energy requirements and environmental damage are kept low (Schinner and Burgstaller, 1989).

The use of autotrophic microorganisms is advantageous in this respect, because no organic carbon source is needed for their growth. On the other hand, heterotrophic microorganisms can be used with higher pHs (alkaline, acid-consuming materials). Another advantage of leaching with heterotrophic microorganisms is the precipitations avoidance when high concentrations of metals and alkaline pHs are present because of the formation of complexes between metals and metabolites. At the same time, complication often reduces the toxicity of metal ions (Avakyan and Rabotnova 1971 & Babich and Stotzky 1980). Because of their capacity for oxidizing the substrate only partially and secreting it again in partly oxidized forms, fungi are of particular interest for leaching. This incomplete oxidation is strongly influenced by the composition of the media and causes the accumulation of organic acids, which are able to extract metals from solid substrates. The most important primary metabolites for leaching is citric acid (Bosecker, 1987).

A pilot plant called 'Biocoil' has been set up in the UK and in Australia (Robinson, 1987). Their biocoil system is claimed to be used in conjunction with bacterial or algal growth to break down toxic wastes and to extract metals from liquids.

Different microorganisms, fungi, algae and higher plants are able to synthesize carotenoids. Production of secondary carotenoids from microalgae surpasses all other sources including yeast and fungi. Production of carotenoids from micro-algae for human consumption is of more interest during the last years, however, more studies should be done to make it economically competitive **(**Bhosale, 2004, Pulz and Gross, 2004 and Spolaore *et al*., 2006**).**

Factors affecting the bio-accumulation of such pigments were fully understood. Carotenoids accumulation by microalgae depend on both nutritional status (Cero'n *et al.,* 2005 and Tittel, 2005) and environmental conditions, such as high light intensity (Bhosale, 2004), type of light (Janhke, 1999**)**. In all cases, the induced algal cells must be used by the log phase growth to avoid the dry weight failure. Also, shifting of photosynthetic metabolism to carotenoids accumulation by lipid biosynthesis should be considered (El-Shafey *et al*., 1999).

In the present work, the green alga *Scenedesmus* sp. (El-Sayed, 2004) was used to study the effect of citrate wastes in the medium on both vegetative

growth and carotenoids accumulation in the presence or absence of some induction factors.

2 - Materials and Methods

2.1. Alga and growth conditions

Polyethylene cylinders containing 2000 ml of nutrient growth media were used for incubating *Scenedesmus* sp. Growth media was composed of ($g.l^{-1}$) 0.56: 0.1:0.01 from urea, phosphoric acid and potassium sulphate; respectively plus 10 ml of citrate wastes. Illumination was provided by day light lamps (12x40w) reflexes from one side. Aeration was performed by compressed air from the upper hold throughout 3mm polyethylene tube. Chemical characteristics of the used waste are listed in Table **1.**

Table 1. Chemical prosperities of citrate waste

pH	E.C $dS.m^{-1}$	O.M	O.C	T.N	C:N ratio
		%			
4.87	4.95	0.80	0.47	0.65	0.73:1
P	K	Fe	Zn	Mn	Cu
%		ppm			
0.013	0.13	395	21	9	13

By the 10^{th} day; as cultures reached their maximum vegetative growth; induction growth was performed by discarding the upper clear layer of growth media and kept the sediment algal bulk to the next induction growth. Here, the induction growth media composed of tap enriched by 50 ml^{-1} of citrate wastes with different induction factors including NaCl (2%) metal $FeSO_4$ and chelated $FeSO_4$ (Table **2**). Sodium acetate was added in both growth periods at a final concentration of 45mM (per each) aiming at enhancing of chlorophyll formation during the vegetative growth period and decomposition of chlorophyll in the presence of salinity (2.0% NaCl) or iron (Fe) as well as carotenoids bioaccumulation during induction.

Table 2. Induction factors used and their concentrations

Control	$FeSO_4$	*Fe(chelate)	NaCl
50m.l^{-1} citrate wastes	50m.l^{-1} citrate wastes + 0.001 g.l^{-1} $FeSO_4$	50m.l^{-1} citrate wastes + 0.001 g.l^{-1} chelated Fe	50m.l^{-1} citrate wastes + 2%NaCl

Wuxal; a micronutrients suspension (3% Fe,3% Zn, 3% Mn and 14%N)

2.2. Growth measurements

Daily measurement of dry weight was routinely carried out by filtering a defined volume of the algal slurry (5-10 ml) over pre-weighted dried membrane filter (0.45 µm). Filters were dried at 105°C for 30 minutes, kept over anhydrous calcium chloride till room temperature and then re-weighted. The difference between weights monitored the net dry weight of the grown alga within define sampling time .

Total chlorophyll was determined based on the methods described by Burnison 1980 on DMSO 95% extract. A modification was done by precipitating the algal biomass over membrane filter after dry weight determination. At 70°C, filters were soaked within 5-10ml of 95% DMSO, filtered and re-extracted if necessary. Chlorophyll absorbance was measured at 666nm and concentration was calculated according to Seely *et al*., 1972. Carotenoids were determined based on the modification done by Boussiba *et al.*, 1992. Filters containing algal biomass were soaked into 5% (w/v) KOH in 30% (v/v) methanol, homogenized and heated in water bath to 70°C for 5 min. Supernatant was discarded and 5 drops of concentrated acetic acid were added to recover carotenoids and the residual was dissolved again by 5-10ml of 95% DMSO. Absorbance was measured at 468nm and concentration was calculated according to Davies 1976. Growth analysis; mainly specific, maximum and average growth rate; was performed using the methods adopted by Pirt, 1973.

3. Result and Discussion

3.1. Vegetative growth

Citrate wastes were previously used to enhance vegetative growth of the green alga *Scenedesmus* sp. in NPK depleted media. Here, full media were used to obtain the biggest bulk which resist algal decay during induction period. Daily yield analysis showed that dry weight accumulation was obviously recorded their log phase by the day eleven.
Chlorophyll content represented the same by the 10^{th} day. Thus, the recommended retention time could be delivered by such time as a result of chlorophyll development curve. Otherwise, the relationship between chlorophyll and carotenoids defining the rate of nutrient consumption or biomass accumulation., where the high ratio during vegetative growth explain the high growth rate which in turn monitored the high nutrient consumption rate with high dry weight accumulation.

As for growth analysis, specific growth rate (µ) showed the long exponential phase as dry weight accumulation, which in turn, explains the high suitability of such media to *Scenedesmus* sp due to its chemical composition or to the adaptation

potential of this alga to the given nutritional conditions. Of this result, maximum growth rate by the third day was 0.29 d^{-1} and the calculated average was 0.13 d^{-1}. A slight decline in the growth rate was observed by the days 5 and 7, which explains the difference between both μ values (maximum and average). As for total chlorophyll, maximum accumulation rate was 0.27d^{-1} with calculated average of 0.16 d^{-1}. The effect of citrate and other like-wastes on algal growth was considerably studied (Ruiz-Marin *et al*., 2010). The main effect on growth enhancement could be attributed to the initial content of some macro and micro-nutrients especially carbon and nitrogen.

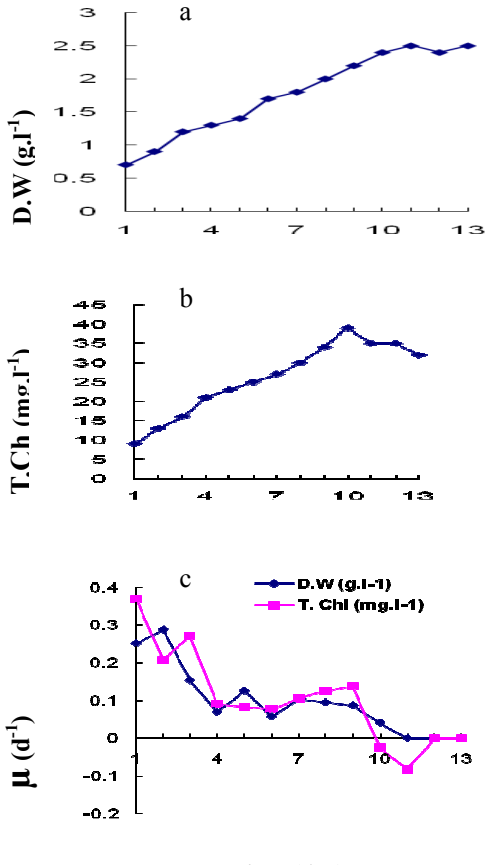

Fig.1. a) Dry weight, **b**) total chlorophyll and **c**) growth rate of *Scenedesmus* sp. grown with 10ml.l^{-1} of citrate wastes

3.2. Induction growth

Different hypothesis on carotenoids accumulation by green microalgae were mentioned. Previous studies on carotenoids accumulation by *Scenedesmus*, *Chlorella* and *Haematococcus* showed that addition of at least 10% of nutrients mainly nitrogen are required to overcome the dry weight accumulation failure (El-Shafey 1999). Here, growth as dry weight was enhanced at first by the addition of citrate wastes even at the higher concentration. The main reason could be ascribed to the presence of organic carbon that allows the fast carotenoids accumulation on the expence of carbohydrate metabolism and green pigments. Addition of extra citrate amount increased the salt potential of the growth medium, even though they are serve as utilizable nutrients as a source of different macronutrients including carbon, nitrogen, phosphorous, potassium and some micronutrients. Growth showed a slight delayed due to such high salt potential, but the growth; in particular; might be goes back to the high initial biomass. Full BG-II medium which received the recommended NPK ratio when enriched by 10 ml.l^{-1} of citrate wastes represented the same afore-obtained result concerning dry weight and total chlorophyll. By the 10^{th} day, all cultures were supplied by extra amount of citrate wastes (40 ml.l^{-1}). Different treatments including salinity (NaCl) and different iron forms represented variable results due to the kind of stress factor used.

When control cultures received only 50 ml^{-1} of citrate waste represented a slight dry weight decreases as compared with the time of citrate addition (following to the 10^{th} day). The decrease on dry weight accumulation due to salting effect was accompanied by chlorophyll decomposition and carotenoids accumulation.

Dry weight: chlorophyll: carotenoids ratio was found as (1.0g : 19.37mg : 7.63mg) at the first cultivation time that explain the high initial biomass as dry weight or total chlorophyll. At the end of vegetative growth, such ratio was found as (1:17.06:13.31) by the same aforementioned respect. Comparing both data showed the slight decrease on chlorophyll content against the increase on carotenoids content. The ratios here are not conditioned by the same rate of dry weight acceleration (Fig.1 and Tab. 3).

The ratio showed the drastic effect of NaCl on growth metabolites, where chlorophyll was drastically decreased and carotenoids were massively increased. Under conditions of 2%NaCl, complete chlorophyll decomposition was observed by Boussiba *et al*., 1992 with *Haematococcus pluvialis*. Here, the inhibitory effect on chlorophyll decomposition by NaCl could be ascribed to the presence of citrate wastes with its initial properties.

Cultures received 50 ml^{-1} of citrate waste plus sodium chloride (2.0%) represented an extra inhibitory effect on dry weight as compared with citrate induced cultures. Chlorophyll decomposition was also engaged with extra carotenoids

accumulation rate. The afore-mentioned ratio was found as (1.0 : 14.52 : 20.64) at the end of induction period. Sodium induced cultures which became nearly orange in color suggesting the extra salting-out potential that affect all the determined algal growth parameters including dry weight and pigments.

Table 3. Dry weight, total chlorophyll and carotenoids ratios of *Scenedesmus* sp grown under different carotenogensis conditions with citrate wastes.

Treatments	Ratio	D.W	T.Ch	T. Car
Control	**V**	**1**	**19.37**	**7.63**
	I	**1**	**17.06**	**13.31**
NaCl (2%)	**V**	**1**	**19.37**	**7.63**
	I	**1**	**14.52**	**20.64**
$FeSO_4$	**V**	**1**	**19.37**	**7.63**
	I	**1**	**3.57**	**44.9**
Fe-Chelate	**V**	**1**	**19.37**	**7.63**
	I	**1**	**12.14**	**37.45**

V = ratio at the end of vegetative growth
I = ratio at the end of induction growth

The effect of salinity against growth metabolism is fully established by many investigations even in halotolerant algae species such as *Dunaliella*. In *Haematococcus pluvialis*, carotenoids accumulation (astaxanthin) was triggered by the addition of 2.0% of NaCl to growth medium (Boussiba *et al*., 1992). Furthermore, the biomass of *Botrycoccus braunii* was increased with the rise of sodium chloride concentration and maximum biomass was achieved in 17mM and 34mM salinity, while phosphate decrease was observed due to its utilization by the alga (Ranga Rao *et al*., 2007). During haloadaptation of such alga their hydrocarbon profile was highly increased, while decreases in protein content and biomass yield. Carbohydrates and lipids remain unchanged may be due to the decrease in photosynthetic rate (Hart *et al*.,1991) or probably due to less adaptability of the organism to high salinity (Vazquez-Duhalt and Arredondo-Vega 1991 and Ben-Amotz *et al*.,1985).

Ferrous sulfate induced cultures were drastically changed and the fast yellow color was observed by the fourth day of induction period. The fast appearing of visual carotenoids suggesting the rapid chlorophyll decomposition, however dry weight was slightly inhibited. The main reason for such results could be ascribed to the effect of ferrous ion on active hydroxyl group (active oxygen species).The ferrous iron form (Fe^{+2}); in particular is known to give rise to free radical formation (especially hydroxyl radicals ($HO^{\bullet}$) via Fenton chemistry. Radical initiation of cellular processes via signal cascade systems is thought to be an integral part of many biological systems and it was suggested that free radicals may play a role in the accumulation of β-carotene in *Dunaliella* (Ben- Amotz & Avron, 1983) and astaxanthin in the yeast *Paffia rhodozyma* (Schroeder & Johnson, 1995). Kobayashi *et al*. (1993) reported that increased astaxanthin formation caused by Fe^{+2} was inhibited by potassium iodide which scavenges $HO^{\bullet}$, suggesting the $HO^{\bullet}$ formed by an iron-catalyzed, where Fenton reaction is required for enhanced astaxanthin biosynthesis in *Haematococcus pluvialis*. As observed early by Droop (1954) and Borowitzka *et al*. (1991), acetate- at small quantities; appeared to be an important carbon source; enhancing both growth and carotenogensis, however, the effect of acetate was concentration dependent. Higher concentrations inhibiting growth, but markedly increasing astaxanthin content per cell. Acetate addition in excess may generate a relative shortage of nitrogen inducing cyst formation and astaxanthin accumulation triggered by a high carbon/nitrogen (C/N) ratio (Kakizono *et al*., 1992). The algal cells seem to reduce their nitrogen uptake and begin to use the cellular nitrogen as in typical N-deficiency, although nitrogen exists in the culture medium. Citrate wastes at high concentration (50 $ml.l^{-1}$) support growth media by high organic carbon and other organic acids which stimulate both vegetative growth and carotenoids accumulation. As mentioned before, in the absence of essential nutrients including nitrogen and phosphorous, increasing of acetate concentrations also rise the salinity potential of the growth media.

Comparing the ratio of dry weight: total chlorophyll: carotenoids showed the highly drastic effect of metal Fe on cell metabolism, where it was found as (1.0: 3.57: 44.9). (Tab. 3 and Fig.2). These Results mean that complete chlorophyll decomposition and extra accumulation of carotenoids are possibility happened. As mentioned before, the stability of dry weight could be attributed to the effect of extra citrate addition.

Cultures received 50 ml^{-1} citrate waste with chelated Fe represented a moderate response and the rate of dry weight accumulation surpasses all other cultures except those of control cultures. Chlorophyll decomposition was slightly inhibited as well as the inhibition of carotenoids accumulation. The massive effect could be oriented as Fe, Na, Fe-chelate and control. It is well known that chelating prevent the free charge of ions and make it easy to penetrate the life cells and also used to remove toxicity of heavy metals even in human body.

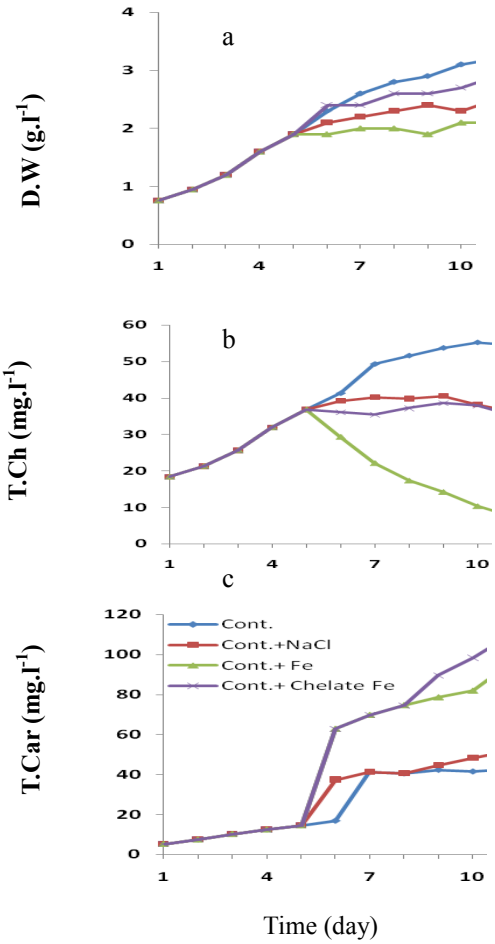

Fig.2. **a**) Dry weight, **b**) total chlorophyll and **c**) growth rate (dry weight and total chlorophyll) of *Scenedesmus sp*. grown on 50ml.l^{-1} of citrate wastes with different induction factors.

Thus, it may be concluded that chelating prevent the high solubility of ferrous in which became slow released ion and delay the free radical generation. In this context, when the chelated iron form was used in this action, no Fenton reaction could be ascribed as mentioned by Borowitzka *et al*., 1991, where he stated that EDTA-chelated $FeC1_3.6H_20$ did not produce any significant differences in astaxanthin formation when added at three different concentrations. Our data could be supported by that obtained by El-Sayed 1999, where, $FeCl_3$ triggered the carotenoids accumulation by *Haematococcus pluvialis*, *Scenedesmus* sp. and *Chlorella* sp. It can be also mentioned that EDTA and other chelating agents neutralize the ionic charges of the used ions which in turn prevent the photo-reaction producing free radicals. The afore-obtained ratio found as (1.0 : 12.14 : 37.14) in which, it explains the inhibitory effect of EDTA against drastic chlorophyll decomposition. Concerning the chelated Fe form used, it is accompanied with the same content of Zn, which minimizes the toxicity of extra Fe addition and also contains 14% of N delivered from urea that enhance vegetative growth and support growth medium by alternative carbon source (El-Sayed *et al*., 2001).

Nitrate concentration played a very important role in the cell division rate and in the accumulation of secondary carotenoids of *H. pluvialis* (Boussiba and Vonshak, 1991). This suggests that the synthesis of astaxanthin requires nitrogen, and most likely reflects the need for continuous synthesis of protein in order to support the massive accumulation of pigments. Nitrogen starvation is an effective way to enhance astaxanthin accumulation in *Haematococcus*. The optimum nitrate concentration to avoid this problem seems to be 0.15 g.l^{-1} (Orosa *et al*., 2005). The ratio chlorophyll-a / total carotenoids could be a good indicator for the physiological status of the culture. Boussiba and Vonshak, 1991 added that in favorable growth conditions, this value was about 4; indicating nutrient replete conditions; however, a value below 3 implied limitation of growth by reduced nutrient supply or another kind of stress. Thus, it is possible to quickly check the N status of the cell as an indicator to astaxanthin accumulation.

4. Conclusion

As it supports growth media by organic carbon and several nutrients, .utilizing of citrate wastes for enhancement of algae vegetative growth is very advisable to reduce production cost and enhancement of the induction growth. In addition, the induction effect suggested to mainly factoring kind and chelating dependent rather than the salt concentration used.

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Corresponding Author

El-Sayed, A.B

Fertilization Technology Department, National Research Centre, Dokki-Cairo, Egypt

bokhair@msn.com

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